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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 20 June 2000 (20.06.00)	
International application No. PCT/GB99/03484	**Applicant's or agent's file reference** 27.9.68443/001
International filing date (day/month/year) 21 October 1999 (21.10.99)	**Priority date** (day/month/year) 21 October 1998 (21.10.98)
Applicant UHLÉN, Mathias et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

22 May 2000 (22.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia RANAIVOJAONA
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

GB9903484

INTERNATIONAL SEARCH REPORT

Intl. Application No
PCT/GB 99/03484

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C07K1/22 C07K14/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B NILSSON ET AL.: "A synthetic IgG-binding domain based on staphylococcal protein A" PROTEIN ENGINEERING., vol. 1, no. 2, 1987, pages 107-113, XP002133892 OXFORD UNIVERSITY PRESS, SURREY., GB ISSN: 0269-2139 the whole document --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	07/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNAT[redacted]L SEARCH REPORT

Inter[redacted]pplication No
PCT/GB 99/03484

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B GUTTE: "Synthetic 63-residue RNase A analogs" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 253, no. 11, 10 June 1978 (1978-06-10), pages 3837-3842, XP002133893 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 the whole document	1-22

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/03484

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-14, 20-21
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 99 03484

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14, 20-21

Present claims 1-15 relate to an extremely large number of possible products and vaguely defined methods. In fact, the claims contain so many variables that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear namely the method based on the products indicated in claim 16-18 and the protein indicated in claims 19 and 21. Moreover the attention of the applicant is drawn to the fact that a library as indicated in claim 5 and a protein as indicated in claim 6 are not distinguishable from any protein obtained by combinatorial chemistry, making a search for a generically defined product devoid of sense.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9.68443/001		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03484	21/10/1999	21/10/1998
International Patent Classification (IPC) or national classification and IPC C12N15/10		
Applicant AFFIBODY TECHNOLOGY SWEDEN AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/05/2000	07.02.01
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Wimmer, G Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03484

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-28	as originally filed			

Claims, No.:

1-26	as received on	19/01/2001	with letter of	19/01/2001

Drawings, sheets:

1/7-7/7	as originally filed

Sequence listing part of the description, pages:

1-5, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☒ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☒ furnished subsequently to this Authority in computer readable form.
   - ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-14, 25-26.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-14, 25-26.

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	15, 17-19
	No:	Claims	16, 20, 22-24
Inventive step (IS)	Yes:	Claims	15, 17-19
	No:	Claims	16, 20, 22-24
Industrial applicability (IA)	Yes:	Claims	15-24
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion.

Only claims 15-19 and 22, as originally filed, were covered by the International Search report; consequently, the no opinion can be given in the International Preliminary Examination Report on subject-matter not covered by the International Search Report.

With facsimile of 19.01.2001, applicants have submitted amended claims 1-26. In this, subject-matter of original claims 15-19 and 22 corresponds to amended claims 15-24, while amended claims 1-14 and 25-26 correspond to original claims not covered by the International Search Report.
Therefore, no opinion on amended claims 1-14 and 25-26 is given with respect to novelty, inventive step, and industrial applicability.

Re Item V

Reasoned statement under Art. 35(2) PCT with regard to novelty, inventive step or industrial applicability.

Novelty under Art. 33(2) PCT.

1) Claim 16 includes a method of affinity separation, or of stabilizing an affinity ligand, wherein one or more of the ligands Asn residues have been modified, and the ligand is a randomized combinatorial protein selected by expression in a surface display library.

   As a consequence of the product-by-process definition of the ligand used in this method, said ligand technically cannot be distinguished from naturally occurring proteins or proteins engineered by any other means. Furthermore, since no limitations are given as to the extent of randomization of the protein in question, *any* protein may therefore represent a randomized form of another protein, and also fulfils the criterium of the modification of a given Asn residue.

   For these reasons, the subject-matter of claim 15 also includes a method of affinity separation, using *any protein* as a ligand.

Since this is a method commonly used in the art, novelty cannot be acknowledged for claim 16.

2) In contrast thereto, the method of claim 15 includes the further step of selecting the randomized affinity ligand by expression in a surface display library.

Again, since the degree of randomization is not limited, proteins used in methods of affinity separation disclosed in the prior art may be viewed to be identical to the modified and randomized proteins of the claim.
However, the claim is formulated in a way to clearly indicate necessary steps of the process. In this, it appears that methods which contain a step of

a) modification of Asn residues to increase protein stability under alkaline conditions,
b) randomization of the protein to modify its binding characteristics, and
c) selecting the randomized affinity ligand by expression in a surface display library

are not envisioned in the prior art listed in the International Search Report.

Claim 15 can therefore considered to be novel, *insofar as the claim is referring back to claim 5.*

However, claim 15 in its current formulation refers back to claims which include product-by-process definitions. For instance, the formulation of claim 1 "an immobilized proteinaceous ligand wherein one or more of its asparagine (Asn) residues have been modified" would also include proteins which have been modified for reasons that are not related to the invention, i.e. the modification of Asn residues to increase protein stability under alkaline conditions.

While the terms claim 15 could therefore also embrace known subject-matter, it is the opinion of the IPEA that none of the prior art, especially the documents cited in the International Search Report, is conflicting with the scope of claim 15. Furthermore, although it is possible that documents exist in the prior art which disclose the use of affinity ligands in which Asn residues have been modified, it should be possible to examine whether these modifications were performed to increase stability under alkaline conditions, or whether these modifications were

merely of accidental nature or for reasons unrelated to the current invention.

Novelty of claim 15 is therefore acknowledged.

3) Although claims 17-19 also refer back to non-novel claim 16, the formulations of the claims allow for a more precise definition of subject-matter. In this, the prior art does not disclose methods of affinity separation using one of the proteins defined in these claims, wherein the proteins have been modified in one ore more Asn residues to achieve protein stability under alkaline conditions, and optionally randomized and selected in a surface display library.
Claims 17-19 are therefore viewed to be inventive.

4) However, the proteins of claims 22-24 are defined through the process for their production. Since thusly created proteins are not distinguishable from the same protein created through other processes, subject-matter e.g. of claim 22 also comprises a protein, derived from e.g. a domain or fragment of a DNA binding protein, in which at least one Asn residue has been modified and wherein part of the protein is randomized.
Since the extent of randomization is however not limited in the claims, *any* protein or polypeptide may be viewed as a modified or randomized version of another polypeptide.
Claim 22 and, for the same reasons, claims 23 and 24, are therefore not novel.

5) Similar objections apply to claim 20. Although this claim does not envision randomization of the protein or fragment, the term "derivatives thereof" is only limited by its suitability "for use in a method of affinity separation". In this, even a completely unrelated protein, which can be used in affinity separation, may be viewed as a "derivative" of ABD wherein "one or more native Asn residues have been replaced".
Novelty of claim 20 therefore cannot be acknowledged.

Inventive Step.

6) Although it was known in the prior art that Asn residues are susceptible to protein degradation under alkaline conditions, none of the prior art documents would lead the skilled person to apply this knowledge to modify proteins, which are used as affinity ligands, in order to increase their stability under the conditions used for washing of and elution from the affinity medium. An inventive step is therefore acknowledged for this method, and accordingly, for claims 15 and 17-19.

Claims

1. A method of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine (Asn) residues has been modified.

2. A method of stabilising an affinity ligand by modifying one or more of its Asn residues.

3. A method of preparing a combinatorial library of protein molecules wherein the protein has been rendered less sensitive to alkaline pH by modification of one or more of its Asn residues before it is randomised.

4. A method of phage display wherein a protein expressed on the phage surface has had one or more of its Asn residues modified in a step separate to any modifications introduced in order to modify binding characteristics of the protein.

5. A method of making a stabilised combinatorial protein comprising the steps of:

a) modification of Asn residues within a protein molecule to increase stability of the protein in alkaline conditions; and

b) randomisation of the protein molecule to modify its binding characteristics.

6. A combinatorial protein wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its Asn residues.

7. A fusion protein comprising a first part wherein one or more naturally occurring Asn residues have been modified and a second part being a randomised protein

molecule selected for its specific binding properties.

8. Use of a protein molecule stabilised by modification of one or more of its Asn residues in surface display or in affinity chromatography.

9. A method or a protein or a use as claimed in any one of claims 1 to 8, wherein one or more Asn residues in said ligand or said protein are replaced with a less alkaline-sensitive amino acid.

10. A method or a protein or a use as claimed in any one of claims 1 to 9, wherein two or more Asn residues are modified.

11. A method or a protein or a use as claimed in any one of claims 1 to 10, wherein all the Asn residues are modified.

12. A method or a protein or a use as claimed in any one of claims 1 to 11, wherein Asn residues on the surface of the three-dimensional structure of the ligand or protein are modified.

13. A method or a protein or a use as claimed in any one of claims 1 to 12, wherein said Asn residues are replaced with an amino acid selected from lysine, aspartic acid and leucine.

14. A method as claimed in any one of claims 1, 2 or 9 to 13, wherein said affinity ligand is a combinatorial protein.

15. A method as claimed in any preceding method claim which further comprises the step of:

c) selecting a randomised affinity ligand by expression in a surface display library.

16. A method as claimed in claim 14, wherein said affinity ligand is a randomised protein selected by expression in a surface display library.

17. A method as claimed in any one of claims 14 to 16, wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

18. A method as claimed in claim 17, wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

19. A method as claimed in any preceding method claim, wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

20. Albumin Binding Protein (ABD) or fragments or derivatives thereof wherein one or more native Asn residues have been replaced by a less alkaline sensitive amino acid, said protein, fragment or derivative being suitable for use in a method of affinity separation.

21. A fusion protein as claimed in claim 7 wherein the first part is ABD and the second part is domain Z (a derivative of the B domain of SPA) or a derivative thereof, said fusion protein being suitable for use in a method of affinity separation.

22. A protein as claimed in claim 6 or claim 7 wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

23. A protein as claimed in claim 22 wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

24. A protein as claimed in any one of claims 6, 7, 22 or 23 wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

25. A nucleic acid molecule encoding a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

26. A host cell expressing a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

PATENT COOPERATION TREATY

by fax and post

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GARDNER, Rebecca
FRANK B. DEHN & CO.
179 Queen Victoria Street
London EC4V 4EL
GRANDE BRETAGNE
FAX NO : +44 20 7206 0700

FILE 6 Fxx.1/pct
12 FEB 2001
ANSI

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Applicant's or agent's file reference 9.68443/001	Date of mailing *(day/month/year)* 07.02.01 **IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03484	21/10/1999	21/10/1998

Applicant
AFFIBODY TECHNOLOGY SWEDEN AB et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel.+49 89 2399-8090

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9.68443/001		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03484	21/10/1999	21/10/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/10		
Applicant		
AFFIBODY TECHNOLOGY SWEDEN AB et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/05/2000	07.02.01
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Wimmer, G Telephone No. +49 89 2399 7347

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03484

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-28 as originally filed

Claims, No.:

1-26 as received on 19/01/2001 with letter of 19/01/2001

Drawings, sheets:

1/7-7/7 as originally filed

Sequence listing part of the description, pages:

1-5, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03484

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-14, 25-26.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 1-14, 25-26.

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03484

1. Statement

Novelty (N)	Yes:	Claims	15, 17-19
	No:	Claims	16, 20, 22-24
Inventive step (IS)	Yes:	Claims	15, 17-19
	No:	Claims	16, 20, 22-24
Industrial applicability (IA)	Yes:	Claims	15-24
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/GB99/03484

Re Item III

Non-establishment of opinion.

Only claims 15-19 and 22, as originally filed, were covered by the International Search report; consequently, the no opinion can be given in the International Preliminary Examination Report on subject-matter not covered by the International Search Report.

With facsimile of 19.01.2001, applicants have submitted amended claims 1-26. In this, subject-matter of original claims 15-19 and 22 corresponds to amended claims 15-24, while amended claims 1-14 and 25-26 correspond to original claims not covered by the International Search Report.
Therefore, no opinion on amended claims 1-14 and 25-26 is given with respect to novelty, inventive step, and industrial applicability.

Re Item V

Reasoned statement under Art. 35(2) PCT with regard to novelty, inventive step or industrial applicability.

Novelty under Art. 33(2) PCT.

1) Claim 16 includes a method of affinity separation, or of stabilizing an affinity ligand, wherein one or more of the ligands Asn residues have been modified, and the ligand is a randomized combinatorial protein selected by expression in a surface display library.

   As a consequence of the product-by-process definition of the ligand used in this method, said ligand technically cannot be distinguished from naturally occurring proteins or proteins engineered by any other means. Furthermore, since no limitations are given as to the extent of randomization of the protein in question, *any* protein may therefore represent a randomized form of another protein, and also fulfils the criterium of the modification of a given Asn residue.

   For these reasons, the subject-matter of claim 15 also includes a method of affinity separation, using *any protein* as a ligand.

Since this is a method commonly used in the art, novelty cannot be acknowledged for claim 16.

2) In contrast thereto, the method of claim 15 includes the further step of selecting the randomized affinity ligand by expression in a surface display library.

Again, since the degree of randomization is not limited, proteins used in methods of affinity separation disclosed in the prior art may be viewed to be identical to the modified and randomized proteins of the claim.
However, the claim is formulated in a way to clearly indicate necessary steps of the process. In this, it appears that methods which contain a step of

a) modification of Asn residues to increase protein stability under alkaline conditions,
b) randomization of the protein to modify its binding characteristics, and
c) selecting the randomized affinity ligand by expression in a surface display library

are not envisioned in the prior art listed in the International Search Report.

Claim 15 can therefore considered to be novel, *insofar as the claim is referring back to claim 5.*

However, claim 15 in its current formulation refers back to claims which include product-by-process definitions. For instance, the formulation of claim 1 "an immobilized proteinaceous ligand wherein one or more of its asparagine (Asn) residues have been modified" would also include proteins which have been modified for reasons that are not related to the invention, i.e. the modification of Asn residues to increase protein stability under alkaline conditions.

While the terms claim 15 could therefore also embrace known subject-matter, it is the opinion of the IPEA that none of the prior art, especially the documents cited in the International Search Report, is conflicting with the scope of claim 15. Furthermore, although it is possible that documents exist in the prior art which disclose the use of affinity ligands in which Asn residues have been modified, it should be possible to examine whether these modifications were performed to increase stability under alkaline conditions, or whether these modifications were

merely of accidental nature or for reasons unrelated to the current invention.

Novelty of claim 15 is therefore acknowledged.

3) Although claims 17-19 also refer back to non-novel claim 16, the formulations of the claims allow for a more precise definition of subject-matter. In this, the prior art does not disclose methods of affinity separation using one of the proteins defined in these claims, wherein the proteins have been modified in one ore more Asn residues to achieve protein stability under alkaline conditions, and optionally randomized and selected in a surface display library.
Claims 17-19 are therefore viewed to be inventive.

4) However, the proteins of claims 22-24 are defined through the process for their production. Since thusly created proteins are not distinguishable from the same protein created through other processes, subject-matter e.g. of claim 22 also comprises a protein, derived from e.g. a domain or fragment of a DNA binding protein, in which at least one Asn residue has been modified and wherein part of the protein is randomized.
Since the extent of randomization is however not limited in the claims, *any* protein or polypeptide may be viewed as a modified or randomized version of another polypeptide.
Claim 22 and, for the same reasons, claims 23 and 24, are therefore not novel.

5) Similar objections apply to claim 20. Although this claim does not envision randomization of the protein or fragment, the term "derivatives thereof" is only limited by its suitability "for use in a method of affinity separation". In this, even a completely unrelated protein, which can be used in affinity separation, may be viewed as a "derivative" of ABD wherein "one or more native Asn residues have been replaced".
Novelty of claim 20 therefore cannot be acknowledged.

Inventive Step.

6) Although it was known in the prior art that Asn residues are susceptible to protein degradation under alkaline conditions, none of the prior art documents would lead the skilled person to apply this knowledge to modify proteins, which are used as affinity ligands, in order to increase their stability under the conditions used for washing of and elution from the affinity medium. An inventive step is therefore acknowledged for this method, and accordingly, for claims 15 and 17-19.

Claims

1. A method of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine (Asn) residues has been modified.

2. A method of stabilising an affinity ligand by modifying one or more of its Asn residues.

3. A method of preparing a combinatorial library of protein molecules wherein the protein has been rendered less sensitive to alkaline pH by modification of one or more of its Asn residues before it is randomised.

4. A method of phage display wherein a protein expressed on the phage surface has had one or more of its Asn residues modified in a step separate to any modifications introduced in order to modify binding characteristics of the protein.

5. A method of making a stabilised combinatorial protein comprising the steps of:

a) modification of Asn residues within a protein molecule to increase stability of the protein in alkaline conditions; and

b) randomisation of the protein molecule to modify its binding characteristics.

6. A combinatorial protein wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its Asn residues.

7. A fusion protein comprising a first part wherein one or more naturally occurring Asn residues have been modified and a second part being a randomised protein

molecule selected for its specific binding properties.

8. Use of a protein molecule stabilised by modification of one or more of its Asn residues in surface display or in affinity chromatography.

9. A method or a protein or a use as claimed in any one of claims 1 to 8, wherein one or more Asn residues in said ligand or said protein are replaced with a less alkaline-sensitive amino acid.

10. A method or a protein or a use as claimed in any one of claims 1 to 9, wherein two or more Asn residues are modified.

11. A method or a protein or a use as claimed in any one of claims 1 to 10, wherein all the Asn residues are modified.

12. A method or a protein or a use as claimed in any one of claims 1 to 11, wherein Asn residues on the surface of the three-dimensional structure of the ligand or protein are modified.

13. A method or a protein or a use as claimed in any one of claims 1 to 12, wherein said Asn residues are replaced with an amino acid selected from lysine, aspartic acid and leucine.

14. A method as claimed in any one of claims 1, 2 or 9 to 13, wherein said affinity ligand is a combinatorial protein.

15. A method as claimed in any preceding method claim which further comprises the step of:

c) selecting a randomised affinity ligand by expression in a surface display library.

16. A method as claimed in claim 14, wherein said affinity ligand is a randomised protein selected by expression in a surface display library.

17. A method as claimed in any one of claims 14 to 16, wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

18. A method as claimed in claim 17, wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

19. A method as claimed in any preceding method claim, wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

20. Albumin Binding Protein (ABD) or fragments or derivatives thereof wherein one or more native Asn residues have been replaced by a less alkaline sensitive amino acid, said protein, fragment or derivative being suitable for use in a method of affinity separation.

21. A fusion protein as claimed in claim 7 wherein the first part is ABD and the second part is domain Z (a derivative of the B domain of SPA) or a derivative thereof, said fusion protein being suitable for use in a method of affinity separation.

22. A protein as claimed in claim 6 or claim 7 wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

23. A protein as claimed in claim 22 wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

24. A protein as claimed in any one of claims 6, 7, 22 or 23 wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

25. A nucleic acid molecule encoding a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

26. A host cell expressing a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

Claims

1. A method of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine (Asn) residues has been modified.

2. A method of stabilising an affinity ligand by modifying one or more of its Asn residues.

3. A method of preparing a combinatorial library of protein molecules wherein the protein has been rendered less sensitive to alkaline pH by modification of one or more of its Asn residues before it is randomised.

4. A method of phage display wherein a protein expressed on the phage surface has had one or more of its Asn residues modified in a step separate to any modifications introduced in order to modify binding characteristics of the protein.

5. A method of making a stabilised combinatorial protein comprising the steps of:

a) modification of Asn residues within a protein molecule to increase stability of the protein in alkaline conditions; and

b) randomisation of the protein molecule to modify its binding characteristics.

6. A combinatorial protein wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its Asn residues.

7. A fusion protein comprising a first part wherein one or more naturally occurring Asn residues have been modified and a second part being a randomised protein

molecule selected for its specific binding properties.

8. Use of a protein molecule stabilised by modification of one or more of its Asn residues in surface display or in affinity chromatography.

9. A method or a protein or a use as claimed in any one of claims 1 to 8, wherein one or more Asn residues in said ligand or said protein are replaced with a less alkaline-sensitive amino acid.

10. A method or a protein or a use as claimed in any one of claims 1 to 9, wherein two or more Asn residues are modified.

11. A method or a protein or a use as claimed in any one of claims 1 to 10, wherein all the Asn residues are modified.

12. A method or a protein or a use as claimed in any one of claims 1 to 11, wherein Asn residues on the surface of the three-dimensional structure of the ligand or protein are modified.

13. A method or a protein or a use as claimed in any one of claims 1 to 12, wherein said Asn residues are replaced with an amino acid selected from lysine, aspartic acid and leucine.

14. A method as claimed in any one of claims 1, 2 or 9 to 13, wherein said affinity ligand is a combinatorial protein.

15. A method as claimed in any preceding method claim which further comprises the step of:

c) selecting a randomised affinity ligand by expression in a surface display library.

16. A method as claimed in claim 14, wherein said affinity ligand is a randomised protein selected by expression in a surface display library.

17. A method as claimed in any one of claims 14 to 16, wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

18. A method as claimed in claim 17, wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

19. A method as claimed in any preceding method claim, wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

20. Albumin Binding Protein (ABD) or fragments or derivatives thereof wherein one or more native Asn residues have been replaced by a less alkaline sensitive amino acid, said protein, fragment or derivative being suitable for use in a method of affinity separation.

21. A fusion protein as claimed in claim 7 wherein the first part is ABD and the second part is domain Z (a derivative of the B domain of SPA) or a derivative thereof, said fusion protein being suitable for use in a method of affinity separation.

22. A protein as claimed in claim 6 or claim 7 wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

23. A protein as claimed in claim 22 wherein said combinatorial protein comprises domain Z (a derivative of the B domain of SPA), or a derivative thereof.

24. A protein as claimed in any one of claims 6, 7, 22 or 23 wherein said affinity ligand comprises Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

25. A nucleic acid molecule encoding a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

26. A host cell expressing a protein as defined in any one of claims 6, 7, 9 to 13 or 22 to 24.

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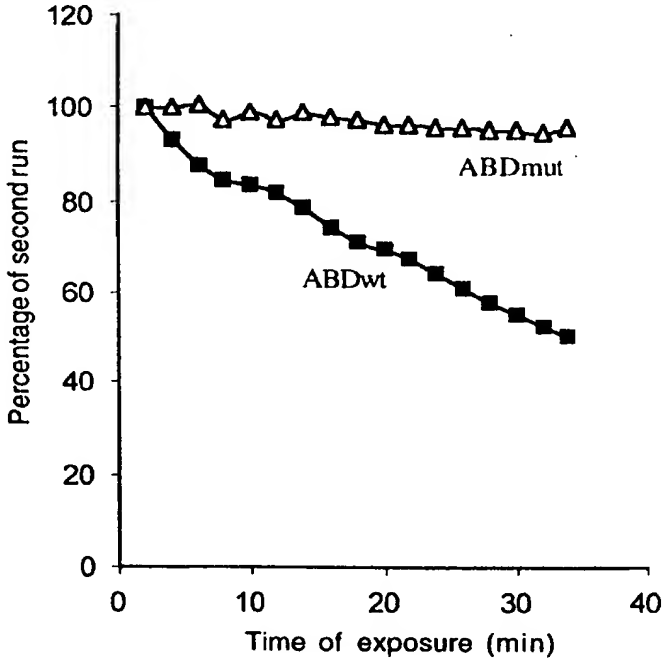
(54) Title: A METHOD OF AFFINITY SEPARATION AND LIGANDS FOR USE THEREIN

(57) Abstract

The present invention relates to methods of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine (Asn) residues has been modified. Methods of making a stabilised combinatorial protein comprising the steps of: a) modification of Asn residues within a protein molecule to increase stability of the protein in alkaline conditions; and b) randomisation of the protein molecule to modify its binding characteristics and combinatorial proteins wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its Asn residues are also described.



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A method of affinity separation and ligands for use therein

The present invention relates to affinity separation and to ligands for use therein.

Affinity separations generally take place on an affinity chromatography column and take advantage of the potentially highly specific nature of interactions involving biomolecules, especially proteins. Other possible separation methods include membrane filtration, two-phase extraction, fluidised beds, expanded beds and magnetic bead separation (Scopes, R.K. Protein Purification, Principles and Practice, 3rd Ed. ISBN 0-387-94072-3). Interactions include protein-protein, protein-nucleic acid, enzyme-substrate, receptor-ligand (e.g. hormone) protein- carbohydrate and protein-metal interactions. Generally, affinity separation is based on the biologically important binding interactions that occur on protein surfaces, such as that between an enzyme and its substrate, between nucleic acid and a DNA binding protein or between antigen and antibody. Either one of a pair of binding partners can be immobilised, e.g. by covalent bonding to an insoluble matrix in an affinity column in order to assay for or purify its binding partner. The very selective nature of interactions between or involving affinity binding molecules, particularly proteins makes them ideal for purification/separation techniques and for many applications, affinity chromatography involving an immobilised protein ligand is preferred over ion-exchange or gel-filtration chromatography.

If a sample is added to a column which carries immobilised on a solid matrix a specific binding partner to a target molecule in the sample, the target molecule will be retained in the column while the majority of the

non-target molecules will simply run through. A solution, typically containing a gradually decreasing pH can be added to the column to wash through firstly any non-specifically bound molecules and finally to elute the target molecule. The target molecule, having the highest affinity for the immobilised ligand, will be the last molecule to be washed off the column and it is therefore the final fractions which will contain the highest concentration of the target molecule and can then be used in a further purification/concentration step or be assayed directly or indirectly for the presence of the target molecule.

Medicine and research in the biochemical and biotechnological fields generally, has created a demand for ever purer samples of organic and biological molecules and thus for strategies which can provide samples of high purity as quickly and cheaply as possible.

As well as satisfying a need to obtain pure samples of proteins and other molecules, affinity separation is important in assaying samples quantitatively and qualitatively for a target molecule. This may be important, for example, when blood or urine samples are being assayed for molecules indicative of a disease such as a metabolic disorder or even the presence of non-naturally occurring substances, narcotics, steroid derivatives etc.

An affinity chromatography column, complete with its solid affinity matrix, can be expensive and clearly it is desirable to be able to re-use the column several times, i.e., to complete a number of runs before it has to be discarded due to reduced ability of the immobilised ligand to bind a target molecule or to a reduced specificity of binding. In order to achieve a set of results which can be directly compared and to clear the column of non-specific and specifically bound molecules before performing another run, it is necessary

to clean the column. The recognised standard for cleaning and sanitizing separation media and systems is NaOH, often in combination with NaCl. An applied 0.1-1.0 M NaOH solution is able to remove viruses, bacteria, nucleic acid, proteins, yeasts, endotoxins, prions and other contaminating agents. The NaOH contact time may vary, between 30 minutes and 1 hour is typical, and removal from the system is monitored by simple in-line pH and conductivity measurements.

However, the ability of the separation media to withstand these rather harsh sanitizing conditions depends on the functional groups of the attached ligand(binding partner), attachment chemistries, and the stability of the base matrices to alkaline conditions. Proteins are sensitive to extreme pH, such as is experienced during NaOH cleaning and, generally speaking, this will adversely affect the effectiveness of protein-based affinity media. Thus, although protein based affinity separation has advantages over ion exchange and gel-filtration chromatography due to its good specificity, these other less specific techniques are not adversely affected by standard cleaning methods.

The sensitivity of proteins to alkaline pH is primarily due to deamidation of asparagine and glutamine residues, particularly asparagine residues. Deamidation of asparagine results in the formation, via a cyclic imide intermediate, of isoaspartate and aspartate, usually in the ratio of 3:1 to 4:1. This reaction does take place at physiological pH but is far faster at alkaline pH such as present in a chromatography column which is being cleaned by an NaOH solution. The isoaspartyl form is characterised by an atypical amide bond between the β-carboxyl of aspartate and the α-nitrogen of the C-flanking amino acid. This results in an extra $-CH_2-$ in the backbone of the protein as well as a free α-carboxyl group. Cleavage of the peptide backbone may occur as a result of the deamidation and

the protein may lose its activity due to a structural change in the whole protein, or merely a small change in a sensitive region such as the active or binding site. The susceptibility of asparagine residues to deamidation is sequence and conformation dependent, Asn residues at Asn-Gly and Asn-Ser sites being particularly vulnerable.

It is a serious problem when an affinity chromatography column has been set up with a protein immobilised on an insoluble support within it and the necessary cleaning process between runs results in reduced efficacy of the system. As well as hastening the end of the absolute useful life of the column, if successive washes decrease the ability of the immobilised protein to capture its binding partner from a sample, comparisons between runs where the concentration of an analyte in a series of samples is measured become meaningless. There is therefore a need for a method of affinity separation wherein the immobilised protein is less susceptible to standard cleaning methods, particularly to alkaline pH.

Thus, in one aspect, the present invention provides a method of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine residues has been modified.

The term "modified" includes deletion of the asparagine residue or replacement of it with a less alkaline-sensitive amino acid, or wherein the asparagine residue has been modified by substitution (i.e. chemical substitution of one or more groups) or other chemical derivitisation, e.g. by a protecting group. Replacement of one or more asparagine residues with a less alkaline-sensitive amino acid is preferred.

By 'affinity separation' is meant any purification or assaying technique which involves the addition of a sample containing a target analyte to a solid which carries on it a specific binding partner to the analyte.

Gravity or other means allows the sample to pass through or across the solid, and the interaction between the analyte and its specific binding partner immobilised on the solid means that the analyte will be retained on the solid while the rest, or most of the rest, of the sample passes through the system. The separation may conveniently be carried out on an affinity chromatography column. The solid is preferably arranged in a column, so that the sample can be added to the top and the non-target part of the sample runs off. An eluant such as a salt solution or a change of pH can be used to displace the specifically bound analyte which can then be collected in a number of aliquots in a controlled manner.

An "affinity ligand" is thus a target-specific binding partner, which can be used in an affinity separation process.

'Analyte' is used to refer to any molecule or fragment of a molecule, proteinaceous or otherwise, in a sample which is capable of binding specifically to the immobilised ligand.

The term 'specific binding partner' may include a molecule or a group of related molecules and any one of these molecules may also bind 'specifically' to one or more other molecules. Thus, the term does not imply that any one immobilised ligand or analyte can have only one binding partner, rather that binding is specific to the extent that most other molecules will not bind with the same affinity or with the same stringency, particularly that other non-target molecules in a given sample will have a much lower affinity. Purification by affinity separation is often of the order of several thousand-fold because of the high affinity between biologically specific binding partners.

All types of affinity separation which include an immobilised proteinaceous ligand can be used in the method of the invention, and suitable matrices (i.e.

solid support) known for use with particular ligands are known in the art, typically based on chromatography using agarose, polacrylamide, silica, polyvinyl styrene, dextran or other polymers. Any of the solid support known in the art for separation or immobilisation processes may be used, as indeed may any of the methods known in the art for attaching molecules such as affinity ligands to solid supports. The proteinaceous ligands are usually attached to the matrix by a coupling agent such as cyanogen bromide, epichlorohydrin, bisoxirane, divinyl sulfone, carbonyl diimidazol, N-hydroxysuccinimide, tosyl/tresyl chloride, epichlorohydrine, carbodiimide, glutaraldehyde, hydrazine, oxirane and also carboxyl or thiol activated matrices and again such coupling agents and coupling chemistries are well known in the art and widely described in the literature (Jansson, J.C. and Rydén, L. Protein purification, 2nd Ed. pp 375-442, ISBN 0-471-18626-0). Various derivatives of matrices which allow straightforward immobilization include CNBr-activated Sepharose 4B, AH-Sepharose 4B and CH-Sepharose 4B and Epoxy-activated Sepharose 6B (Pharmacia).

The proteinaceous affinity ligand may be a molecule having a protein component, which may function as a "specific binding partner" as defined above. Thus, glycoproteins or protein-lipid complexes or indeed proteins with prosthetic groups may be used as the affinity ligand which is modified according to the present invention. Protein molecules are, however, preferred. The term "protein" is used herein broadly to include any molecule having a polypeptide or peptide structure. In other words, as used herein, a "protein" is made up of a chain of amino acids, and the term does not imply any particular conformational (i.e. tertiary structure) or other requirement.

Suitable amino acids to replace asparagine include any of the other 19 standard naturally occurring amino

acids, although cysteine and glutamine would not be preferred. Preferred substituting amino acids include lysine, aspartic acid and leucine. Non-naturally occurring amino acids and amino acid derivatives which are well known to the man skilled in the art could also be used to replace asparagine residues. Significant improvements in the stability of the immobilised protein and thus the efficacy of the column after cleaning can be observed when just one asparagine is substituted by a less alkaline sensitive residue but preferably 2, 3 or more, or even all of the asparagine residues are substituted.

A "less alkaline-sensitive amino acid" is one which is less susceptible to degradation under alkaline conditions than Asn, when compared using techniques, methods and conditions known in the art. Such conditions may, for example, be the column washing conditions discussed above, or any other alkaline conditions used in the art to study protein stability or degradation e.g. deamidation as discussed above. A "less alkaline-sensitive" amino acid may thus be any amino acid other than Asn, and more preferably other also than Gln and Cys. Conveniently, alkaline-sensitivity may be compared by replacing a given Asn residue in a protein molecule with a substitute amino acid, or by chemically modifying or derivatising the said Asn residues, and then comparing stability in alkaline conditions (e.g. column wash conditions) with the unmodified protein.

As indicated above, the sensitivity of a particular asparagine residue to deamidation will depend on the configuration of the protein, it being particularly important to replace those residues which are on the surface of the three dimensional structure of the protein and thus particularly exposed to the alkaline conditions. It is the overall stability of the ligand which is of most importance and it is desirable to

retain the specificity of the interaction between the immobilised ligand and its binding partner, therefore, preferably it is the asparagine residues not involved in the ligand-analyte interaction which are replaced.

The "asparagine modified" proteinaceous affinity ligand (hereinafter the "modified protein") may be made by any method known to the skilled man. Standard techniques for site-directed mutagenesis of nucleic acids are described, for example, in the laboratory manual entitled Molecular Cloning by Sambrook, Fritsch and Maniatis. A preferred technique involves PCR mutagenesis, where primers which incorporate the necessary mis-match base pairs to generate the desired mutations are used in a first round of PCR. In the second run, the fragments from the first run are mixed and the polymerase is able to fill in the strands. The resulting double stranded fragment can be ligated into a plasmid which is then used to transform *E. coli*. The protein can by synthesised *in vitro* without using a biological host and in this case non-naturally occurring amino acids can be introduced.

As well as the many general uses of affinity separation indicated previously, of particular interest in the present case is the use of affinity separation with ligands which have been made by randomisation (random mutagenesis) of a particular protein to generate ligands with novel, modified or enhanced binding characteristics. These proteins are referred to as combinatorial proteins. Such a technique typically involves random mutagenesis of a target protein, expression of the full library of these variants, e.g. on the surface of filamentous bacteriophage, followed by selection of a protein exhibiting the desired binding characteristics, this selection typically involving a binding reaction between the variant protein and an immobilised ligand (binding partner) i.e. target molecule for the protein, e.g. target analyte. The

mutagenesis is random in that the resulting amino acid encoded by any particular codon is not generally pre-determined but the positions where mutations are to be introduced are generally identified in advance. The mutagenesis may involve amino acid substitution deletion, or addition (e.g. insertion).

The use of an expression system such as surface display on phage provides a crucial link between genotype and phenotype; there is a self-contained unit which can be selected on the basis of its specific binding interactions and which also carries the nucleic acid encoding for the protein responsible for the observed binding characteristics. This enables expression in useful amounts of the protein selected for its binding characteristics, such expression typically taking place in a transformed bacterial host.

The protein, selected by its ability to bind to an immobilised ligand (e.g. a desired target molecule (analyte)), is then itself used in affinity separation (i.e. as the affinity-ligand). It is immobilised and used to purify or assay for a target molecule in a sample, typically the same ligand which was used to select the protein in the first place. In this way, a protein from a library of variants can be selected for its ability to bind e.g. insulin, using a column with insulin immobilised on a matrix therein and the selected protein can then be used to test samples for the presence of insulin.

As well as testing for the presence of a target molecule, the affinity separation methods of the invention provide excellent purification methods, yielding samples of a target molecule having good purity. In particular, the methods of the invention will still produce pure samples after many cycles of the separation system, e.g. after many runs, with washing, of a column. Such samples produced by the methods of the invention constitute further aspects of the

invention.

If a protein is stabilised by replacing one or more of its asparagine residues with a less alkaline sensitive amino acid prior to the randomisation and selection steps described above, then its useful life as an affinity ligand will be extended due to its ability to withstand the harsh conditions such as high pH which are experienced when an affinity column is washed between runs.

Therefore, a further aspect of the present invention comprises a combinatorial protein wherein in a step separate from the randomisation step (i.e. the step used to generate the combinatorial protein by randomisation of an "origin" or "source" or "starting protein"), the stability of the protein in alkaline conditions has been increased by modifying one or more of its asparagine residues (preferably by replacing one or more of its asparagine residues with a less alkaline sensitive amino acid).

Nucleic acid molecules encoding such a protein as well as cells expressing the protein constitute further aspects of the present invention.

The randomisation may itself result in substitution of asparagine residues but this aspect of the invention is concerned with proteins which have also been specifically modified to replace one or more asparagine residues in order to increase stability. The protein can be stabilised before or after the randomisation or at the same time but preferably the stabilising modifications will be introduced before the randomisation takes place. If stabilisation is performed before randomisation, it need only be performed once, and by selecting from the library of randomised variants against a number of ligands, several stabilised proteins with different binding characteristics can be obtained. If the stabilisation is performed after selection for a particular binding

affinity, then there is a risk that some affinity would be lost as a result of the stabilising substitutions.

Techniques for construction of a combinatorial library of protein molecules and subsequent selection to obtain proteinaceous ligands having desired binding characteristics are known in the art (Nygren, P. and Uhlén, M. Current Opinion in Structural Biology (1997) 7: 463-469). Generally, a protein molecule, perhaps having intrinsic beneficial properties such as temperature or pH insensitivity, is used as a scaffold and a combinatorial library is then constructed via random but targeted amino acid substitutions (or other mutations) of that protein molecule, in order to produce a library of molecules having different binding characteristics. Surface residues are generally targeted for random mutagenesis.

Suitable protein scaffolds may simply be linear peptides but preferably the scaffold will possess a folded three dimensional structure which has the potential for higher affinities and is less susceptible to proteolytic degradation. Rather than designing a scaffold *de novo*, naturally existing proteins or domains are usually selected for further engineering. For the avoidance of doubt, it is to be noted that throughout this specification the word "protein" is used to refer to whole protein molecules as well as domains or fragments thereof, polypeptides or peptides.

The choice of protein scaffold depends on several parameters including an ability to be effectively expressed in a desired host organism e.g *E. coli* when the randomised protein is to be displayed as a fusion protein with a filamentous phage coat protein. The protein should also comprise sufficiently large regions on its surface which are tolerant to substitution (or insertion or deletion etc.) without losing the overall three dimensional structure. If the library is to be produced synthetically, a small overall size is a

prerequisite. Where the selected scaffold protein has a binding function, amino acid residues involved in that interaction may be a target for randomisation. Randomisation may be performed in order to enhance known binding properties or to develop ligands with new specificities.

Suitable scaffold molecules are discussed in Nygren et al. (1997) and include cyclic peptides having 40 or more residues in a constrained sequence, immunoglobulin-like scaffolds including Fv or single-chain (scFv) domains, bacterial receptors such as the 58-residue one-domain *Staphylococcal* protein A (SPA) analogue Z (the "Z Domain" being a derivative of the B domain of SPA), or other domains or analogues of SPA, DNA-binding proteins particularly zinc fingers and protease inhibitors. All these molecules can be stabilised by substituting one or more native asparagine residues with a less alkaline sensitive amino acid before a combinatorial library of the pre-stabilised protein is made.

Of particular interest is the bacterial receptor domain Z (Nord, K., Nilsson, J., Nilsson, B., Uhlén, M. and Nygren, P. Protein Engineering (1995) 8, 6, 601-608). This paper by Nord *et al.* describes a suitable method of constructing a combinatorial library of protein molecules which can be applied to a range of scaffold molecules. The method described is solid-phase-assisted and based on the stepwise assembly of randomised single-stranded oligonucleotides.

Selection from a generated protein library can be performed in a number of different ways known in the art, including bead immobilised libraries (McBride, J.D., Freeman, N., Domingo, G.J. and Leatherbarrow, R.J., J. Mol. Biol. [1996] 259: 819-827), fusions to DNA-binding proteins (Schatz, P.J., Biotechnology [1993] 11, 1138-1143) and when displayed on bacteria (Lu, Z., Murray, K.S., Van Cleave, V., LaVallie, E.R., Ståhl, M.L. and McCoy, J.M. Biotechnology [1995], 13 366-372)

or phage (Clackson, T. and Wells, J. TIBTECH (1994) 12, 173-183) as well as yeast cells, (Boder, E.T. and Wittrup, K.D., Nature Biotechnology (1997) 15, 553-557) and in viral systems (Ernst, W., Grabher, R., Wegner, D., Borth, N., Graussauer, A. and Katinger, H. Nucleic Acids Research (1998) 26, 1718-1723 and Grabher, R., Ernst, W., Doblhoff-Dier, O., Sara, M. and Katinger, H. BioTechniques (1997) 22, 730-735).

International Patent Application, publication No. WO 95/19374 describes the generation of a combinatorial library of Z-variants, see in particular Example 4. This application discusses on page 12 the advantages of the Z domain as an affinity ligand in the purification of recombinant proteins, as it is relatively stable in the harsh environment of an affinity column during cleaning. The present invention offers further benefits in that the Z domain can be stabilised to alkaline pH by replacement of asparagine residues. According to the present invention it is proposed to stabilise the native Z domain before the combinatorial library is prepared.

Thus in a further aspect, the present invention provides a method of preparing a combinatorial library of protein molecules wherein the protein has been rendered less sensitive to alkaline pH by modification of one or more of its asparagine residues before it is randomised (preferably by replacement of one or more of its asparagine residues).

In a yet further aspect of the present invention is provided a method of phage display wherein a protein expressed on the phage surface has had one or more of its asparagine residues modified (preferably by replacement with a less alkaline sensitive residue) in a step separate to any modifications introduced in order to modify binding characteristics of the protein.

A further aspect of the present invention comprises a method of making a stabilised combinatorial protein comprising the steps of:

a) modification of asparagine residues within a protein molecule to increase stability of the protein in alkaline conditions; and

b) randomisation of the protein molecule to modify its binding characteristics.

Modification in step a) will preferably comprise replacement of asparagine residues with other less alkaline sensitive amino acids, Step a) is preferably carried out before step b).

As well as a protein molecule which has been randomised and stabilised, the present invention also relates to fusion proteins comprising a stabilised part and a randomised part, such proteins being useful in certain applications. In particular, this technique enables the development of a stable framework which can have attached thereto a variable region with specific binding characteristics. The variable region can be prepared by randomisation techniques as discussed above and a protein molecule having desired binding characteristics can be selected from the library of variants, for example by phage display and affinity chromatography. This randomised protein can then be expressed as a fusion protein, e.g. in *E. coli*, together with a protein molecule which has already been engineered to improve its stability to alkaline conditions by replacing one or more of its asparagine residues with other less alkaline sensitive residues.

The fusion protein can be used as an immobilised ligand in affinity chromatography, giving the benefits of a generally stable molecule provided by the framework part as well as a pre-selected binding affinity provided by the randomised part. Such a system may enable the use of a small protein molecule during the randomisation and selection stage as important protein-like characteristics (as opposed to peptide characteristics) are provided by the stabilised part. The same stabilised part can be used together with a variety of

different randomised molecules and vice versa. Suitable protein molecules to perform as the stabilised framework part of the fusion peptides include Albumin Binding Protein (ABD); the bacterial receptor domain Z is a suitable molecule for randomisation in this context.

Thus the present invention also provides a fusion protein comprising a first part wherein one or more naturally occurring asparagine residues have been modified (preferably replaced by an amino acid residue less sensitive to high pH) and a second part being a randomised protein molecule selected for its specific binding properties.

Nucleic acid molecules encoding such a protein as well as cells expressing the protein constitute further aspects of the present invention.

A further aspect of the present invention comprises a method of stabilising an affinity ligand by modifying one or more of its asparagine residues (preferably the affinity ligand is stabilised by replacing one or more of its asparagine residues with an amino acid residue less sensitive to alkaline pH).

A still further aspect comprises the use of a protein molecule stabilised by modification of one or more of its asparagine residues in surface display or in affinity chromatography.

The term 'surface display' refers to the technique involved in selection of a protein from a library of molecules which are presented (displayed) in a manner which enables differentiation between the protein molecules on the basis of their binding characteristics. Surface display is typically performed on the surface of filamentous bacteriophage (phage display) but display can also be on the surface of bacteria, yeast cells or using viral systems. Any "surface display" technique known or proposed in the art, may be used according to the present invention.

A preferred affinity ligand for use in the methods

of the invention is Albumin Binding Protein (ABD), a protein domain with affinity towards human serum albumin (HSA). It is derived from a cell wall anchored bacterial receptor protein from *Streptococcus* G148. It is of particular use as an affinity ligand for the purification of human serum albumin (HSA). The wild type sequence of this protein has four asparagine residues, enhanced stability is observed when just one of these residues is substituted but preferably all four residues are replaced by less alkaline sensitive residues. The wild type amino acid sequence of ABD is:
LAEAKVLA**N**RELDKYGV-SDYYK**N**LI**NN**AKTVEGVKALIDEILAALP

the asparagine residues have been indicated in bold. The hyphen simply indicates that other molecules in the same family have an additional amino acid in this position. This sequence excludes the 19 amino acid N-terminal 'tail' shown in Figure 3. Throughout the text particular amino acids of ABD are identified by their position in the full sequence (including the 19 amino acid 'tail') as presented in Figure 3. Thus, the first asparagine residue of ABDwt which may be stabilised is in position 28 (Asn_{28}). Stabilised versions of ABD for use in the methods of the invention may incorporate some, all or none of the 19 amino acid N-terminal tail.

Stabilised ABD may, as discussed above, be further subjected to randomisation to create a protein (i.e. a combinatorial protein) having e.g. modified binding characteristics for HSA and/or that is able to bind any target of choice and also retains affinity towards HSA. The randomisation may involve mutagenesis of the same, or preferably different, residues modified in the stabilisation step. Such derivatives of ABD (e.g. mutants created by random or directed mutagenesis) are also included within the scope of the invention.

Thus a further aspect of the present invention is Albumin Binding Protein(ABD) or fragments or derivatives

thereof wherein one or more native asparagine residue have been replaced by a less alkaline sensitive amino acid. Nucleic acid molecules encoding such a protein as well as cells expressing the protein constitute further aspects of the present invention.

In a particularly preferred embodiment Asn_{28} is replaced by leucine, Asn_{42} by aspartic acid, Asn_{45} by aspartic acid and Asn_{46} by lysine (referred to as ABDmut herein). 'ABDmut' is used to refer to proteins incorporating some, all or none of the 19 amino acid N-terminal tail. Typically, if ABDmut is part of a fusion protein, e.g. with domain Z, then only part or none of the N-terminal tail will be present. Asparagine is the most alkaline sensitive amino acid and so any other amino acid residue could be used to replace it and an increase in stability would be expected. It may be of assistance to compare the sequences of other homologous proteins, which are found in other species or perform the same role, to identify suitable amino acids to be used in the substitution.

In a method suitable for the stabilisation of other affinity ligands, the modifications were introduced by PCR mutagenesis. Firstly mismatched primers are used to introduce the mutations and then the fragments from the first run of PCR are mixed and the polymerase allowed to fill in the strands. The double stranded fragment of ABD incorporating the modifications is cleaved with restriction enzymes and ligated into a plasmid restricted with the same enzymes, which is then used to transform *E. coli*, the expression system for the protein.

Preferably, the affinity ligand purified (i.e. obtained) according to the methods of the invention retains more than 80%, preferably more than 95%, of its normal binding capacity after 20 rounds of treatment with 0.5M NaOH.

The invention will be further described in the

following non-limiting Examples in which:

Figure 1 is a schematic description of the PCR-mutagenesis step according to the invention;

Figure 2 shows the cloning of the gene construct;

Figure 3 is the amino acid sequence of wild type ABD and a mutated alkaline resistant variant of it, the full sequence of the mutated variant is designated herein as SEQ ID NO. 1;

Figure 4 shows an SDS-PAGE (4-20%) under reducing conditions. Lanes 1 and 2 show purified ABD and ABDstab respectively. The third lane shows a molecular weight marker (from top 94, 67, 43, 40, 20.2 and 14.4 kDa respectively). In order to check the selectivity of the mutated ABD, E. coli disintegrate was spiked with HSA (lane 4) and loaded onto the column. Lanes 5, 6 and 7 show the eluted material after 2, 8 and 15 rounds of NaOH treatment. Lane 8 shows an HSA reference;

Figure 5 shows Sensorgrams from the BIACore showing the binding characteristics of (A)ABDwt and (B)ABDmut before and after treatment with 0.5M NaOH for 24 hours;

Figure 6 is a diagram showing the capacity of two affinity matrices, ABDwt and ABDmut respectively, after several rounds of treatment with 0.5M NaOH; and

Figure 7 shows sensorgrams from the Biacore experiment described in Example 10 showing the binding characteristics of ABDmut and the three different triple-mutants thereof, Response Unit (RU) v Time (S); Δ ABDmu; ▲ 29-30-33A; □ 54-57-58 A; ■ 22-25-26 A.

Throughout the Examples ABDstab and ABDmut are used synonymously.

Example 1 - Cloning

As a template for the PCR mutagenesis pTrpABDT1T2 was used (Kraulis *et al.* (1996) FEBS Lett. **378**, 190-194). The plasmid encodes the gene for ABDwt under control of the tryptophan promoter. The PCR mutagenesis was

performed in two steps. In the first PCR the mutations was introduced and in the second round of PCR, the fragments from the first run were mixed and the polymerase was allowed to fill in the strands (Figure 1). The primers used in the first PCR were:
5'-ACGTAAAAAG GGTATCTAGA ATTATGAAAG C-3' (SOHO8)(SEQ ID NO. 2)
3'-CAGAATCGAG ACTCTCTCGA GCTGTTTATA CC-5' (SOHO6)(SEQ ID NO. 3)
5'-GAGAGAGCTC GACAAATATG GAGTAAGTGA CTATTACAAG GATCTAATCG ATAAAGC-3' (SOHO7)(SEQ ID NO. 4)
3'-CCGCCTACTC TCTTCTAAAA GTCG-5' (T1T2)(SEQ ID NO. 5)

The double stranded fragment was cleaved with Xba I and Pst I and ligated in to pTrpABDT1T2 restricted with the same enzymes (Figure 2). The ligation mixture was used to transform *E. coli*, strain RRIΔM15. After confirming the size of the inserts by PCR, the sequence was verified by cycle sequencing (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting plasmid was denoted pTrpABDmutT1T2.

Example 2 - Expression and purification

E. coli cells harboring the plasmid encoding ABDmut and also the wild type ABD as a reference were grown over night in shake flasks containing 500 ml Tryptic Soy Broth (30 g/l) supplemented with yeast extract (Difco, USA) (5 g/l) and kanamycin monosulphate (50 mg/l). Since the ABD gene is under control of the tryptophan promoter, the m-RNA production starts when the amino acid is missing in the growth medium. Cells were harvested after 20 hours by centrifugation, 4000xg in 10 minutes. After resuspending the cells in 30 ml TST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) they were fractured by sonication. After that a centrifugation step was performed, approximately

40,000xg in 20 minutes. The supernatant was filtered (0.49 μm). The soluble protein was isolated by affinity chromatography on human serum albumin (HSA) Sepharose as described by Stahl *et al.* (1989) J. Immunol. Meth. **124**, 43-52. The protein content in eluted fractions was measured by absorbance at 280 nm and relevant fractions were collected and lyophilized. Both proteins are of high purity after a single step purification and migrate in accordance with their molecular masses.

Example 3 - Binding characteristics before and after NaOH-treatment

In order to examine the stability of ABDwt and ABDmut, the binding characteristics were analysed before and after treatment with 0.5 M NaOH in room temperature for 24 hours. Both ABDwt and ABDmut were solubilized in 1xHBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20) to a concentration of 800 nM and analyzed on BIAcore 2000 (Biacore AB, Uppsala, Sweden). In order to determine the activity change after exposure to a very alkaline environment, the proteins were solubilized in 0.5 M NaOH. After incubation in NaOH for 24 hours, the samples were run through a NAP-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 30 mM NH_4Ac. The protein containing samples were lyophilized and resolubilised in 1xHBS to a concentration of 800 nM in order to be analyzed in the BIAcore 2000.

BIAcore analysis

A BIAcore 2000 instrument (Biacore AB) was used for real time affinity analysis. HSA and IgG were immobilized on two different surfaces of a CM5 sensor chip by amine coupling to the carboxylated dextran layer. This coupling was done according to the manufacturer's recommendation (Biacore AB). The IgG surface was used

as a control. The samples were run through a 0.45 μm filter and injected over the surface in a random order at a flow rate of 5 μl/min. The results clearly show that ABDwt loses the affinity towards HSA during exposure to alkaline solution whereas the mutated variant that lacks four asparagine residues, has the affinity preserved. The sensorgrams are shown in Figure 5.

Also, an analysis of the kinetic parameters was performed for the proteins that still remained active. This was done by analysing the binding behaviour in different concentrations and thereafter calculating the binding constants by using BIAevaluation 2.1 software (BIAcore AB). The concentrations used were 40-220 nM for ABDwt, 200-600 nM for ABDmut and 800-1250 nM for the NaOH treated ABDmut. The results are shown in table 1 below.

Table 1

	K_{on} (1/Ms)	K_{off} (1/s)	K_{aff} (1/M)
ABDwt	$9.0x10^4$	$1.3x10^{-3}$	$6.9x10^7$
ABDmut	$1.8x10^4$	$1.2x10^{-3}$	$1.5x10^7$

K_{on} represents the rate at which the proteins associate, K_{off} the dissociation rate and K_{aff} is the affinity constant which is calculated as the ratio between K_{on} and K_{off}.

Example 4 - Preparation of affinity matrices

In order to evaluate the usability of the stabilized HSA-binding protein in biotechnology, two affinity chromatography matrices were prepared; one with the unmutated protein (ABDwt) as a control and the other with the mutated ABD (ABDmut). The matrix used was

Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) with carbodiimide CMC (Sigma Aldrich, Sweden) as the coupling reagent. ABDwt (4.5 mg) and ABDmut (8.5 mg) were dissolved in 9 ml water separately. 3.5 and 5.0 ml gel was added to ABDwt and ABDmut solutions respectively. Finally 0.38 g of CMC was added to the solutions and they were incubated at room temperature over night. The gels were packed on HR columns (d=5 mm, h=43 mm) and pulsed with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 in order to inactivate the matrix and 0.1 M NaAc, 0.5 M NaCl, pH 4 in order to wash unbounded proteins out. After this, the columns were ready to use for affinity purification of HSA.

Example 5 - Retained selectivity

To investigate if the selectivity of the mutated protein towards HSA was retained after exchanging four amino acids, a capture experiment was done. A culture of *E. coli* cells grown over night was fractured by sonication, centrifuged at 40,000xg and filtered through a 0.45 μm filter. The soluble fraction of the *E. coli* lysate was mixed with pure HSA and the mixture was applied on the ABDmut affinity column. After washing the column, the bound material was eluted by lowering the pH to 2.8 as described by Stahl et al., (1989) supra. As can be seen in Figure 4, the selectivity is retained in the mutated variant of ABD.

Example 6 - Alkaline resistant affinity columns

In order to explore the difference between the ABDwt column and the ABDmut column in respect of stability against alkaline treatment, both columns were repeatedly washed with 0.5 M NaOH. By using the AKTA explorer the columns were loaded with HSA, the protein was eluted by lowering the pH (Ståhl et al., (1989) supra) and finally

both columns were washed with NaOH. This cycle was repeated 15 times and the total time of exposure exceeded 6 hours. The flow rate used was 60 cm/h and the eluted material was collected and analyzed in each round. In Figure 6 the decrease in capacity is plotted against the NaOH exposure time. As can be seen in the figure, ABDwt is losing the activity quite fast while the mutated variant is keeping the activity throughout the experiment. These results corroborate with the BIACore data, the mutated variant is much more stable against cleaning-in-place (CIP) treatment than the wild type molecule.

Example 7 - Production and purification of Z-ABDstab

A frozen E. coli RRIΔM15 culture, harbouring the expression vector pTRPZABDstabT1T2 (see Example 8) was used to inoculate 20 ml Tryptic Soy Broth (30 g/l) (Difco, Detroit, MI, USA) supplemented with 5 g/l yeast extract (Difco) and 50 mg/l kanamycin monsulphate (Labkemi, Stockholm, Sweden). 10 ml of the over night culture was used to inoculate 500 ml of fresh medium. The cells were allowed to grow at 37°C and expression of the proteins was induced at mid-log phase (A_{600nm}=1) by adding 3-β-indole acrylic acid (SIGMA-Aldrich, Stockholm, Sweden) to a final concentration of 25 mg/ml. After 24 hours the cells were harvested by centrifugation at approximately 5000g for 10 minutes followed by resuspension in TST buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.25 mM EDTA, 0.05% Tween20). The cells were disintegrated by sonication (Vibracell, Sonics & Materials, Danbury, CT, USA) and centrifuged at 30000g for 20 minutes. The supernatant was filtered through 0.45 μm filters (Millipore Corp., Bedford, MA, USA) and subjected to HSA affinity chromatography (Nygren et al. 1988). The amount of eluted protein was estimated by absorbance measurements using the specific

absorption constant, a (L/g*cm), and relevant fractions were lyophilized.

Example 8 - Genetic construction of the fusion protein Z-ABDstab

Plasmid pTRPZABDstabT1T2 was constructed from pTRPABDmutT1T2. A gene fragment of 233 base pairs, encoding the Z domain was isolated from pRIT45 (Nilsson et al. (1994) Eur. J. Biochem. **224**, 1038-108) by XbaI-EcoRI-digestion and ligated into the pTRPABDmutT1T2, which has previously been cut with the same restriction endonucleases. The ligation mixture was used to transform E. coli, strain RRIΔM15. The resulting plasmid contained a gene encoding the fusion protein Z-ABDstab and was denoted pTRPZABDstabT1T2.

The DNA sequences of ABDstab and Z-ABDstab (including the region coding for the 19 amino acid N-terminal tail) are as follows:

ABDstab (SEQ ID NO. 11)

ATGAAAG CAATTTTCGT ACTGAATGCG CAACACGATG AAGCCGTAGA CGCGAATTCA TTAGCTGAAG
CTAAAGTCTT AGCTCTGAGA GAGCTCGACA AATATGGAGT AAGTGACTAT TACAAGGATC TAATCGATAA AGCCAAAACT GTTGAAGGTG TAAAAGCACT GATAGATGAA ATTTTAGCTG CATTACCTTA A

Z-ABDstab (SEQ ID NO. 12)

ATGAAAG CAATTTTCGT ACTGAATGCG CAACACGATG AAGCCGTAGA CAACAAATTC AACAAAGAAC
AACAAAACGC GTTCTATGAG ATCTTACATT TACCTAACTT AAACGAAGAA CAACGAAACG CCTTCATCCA AAGTTTAAAA GATGACCCAA GCCAAAGCGC TAACCTTTTA GCAGAAGCTA AAAAGCTAAA TGATGCTCAG GCGCCGAAAG TAGACGCGAA TTCATTAGCT GAAGCTAAAG TCTTAGCTCT GAGAGAGCTC GACAAATATG GAGTAAGTGA CTATTACAAG GATCTAATCG ATAAAGCCAA AACTGTTGAA GGTGTAAAAG CACTGATAGA TGAAATTTTA GCTGCATTAC CTTAA

Example 9 - Genetic construction of three triple mutants of the ABDmut domain

Examples 9 and 10 show that it is possible to randomise a surface on the already stabilised ABD-molecule, for example a surface that does not take part in the HSA-binding and thereby create a molecule that is able to bind any target of choice and also retains affinity towards HSA.

Based on the results from an alanine scan experiment to map the binding site for HSA, gene constructs were made encoding ABDmut (see Example 1) domains containing additional alanine substitutions. These were introduced at positions in the domain pointing away from the identified HSA binding motif, which had been located to the first and second helix and the loop in-between. Three different plasmid gene constructs were assembled by PCR based mutagenesis, each encoding ABDmut protein variants containing three alanine substitutions: pTrpABDmut22-25-26 containing alanine substitutions at positions 22, 25 and 26 of the ABDmut domain; pTrpABDmut29-30-33 containing alanine substitutions at positions 29, 30 and 33 of the ABDmut domain, and pTrpABDmut54-57-58 containing alanine substitutions at positions 54, 57 and 58 of the ABDmut domain. As a template for the PCR mutagenesis plasmid pTrpABDmutT1T2 was used (see Example 1). The plasmid encodes the gene for ABDmut under transcriptional control of the tryptophan promoter. Exchange of amino acids 22, 25 and 26 for alanine was done by an ordinary PCR mutagenesis using the primer T1T2 (see Example 1) and LIMA 12:

5'-GTAGACGCGA ATTCATTAGC TGCTGCTAAA GCAGCTGCTC TG-3' (LIMA12)(SEQ ID NO. 6)

The PCR mutagenesis for the other two constructs was

performed in two steps. In the first PCR amplification the mutations were introduced and in the second PCR, the fragments from the first PCR amplification were mixed and subjected to *Taq* DNA polymerase assisted double strand DNA synthesis (the same strategy as in Figure 1), followed by amplification using flanking primers (SOHO8 and T1T2 (see Example 1)). The primers used in the PCR for exchange of amino acids 29, 30 and 33 for alanine were:

5'-ACTCCATATG CGTCGAGCGC TGCCAGAGCT-3' (LIMA13)(SEQ ID NO. 7)

5'-AGCGCTCGAC GCATATGGAG TAAGTGACT-3' (LIMA14)(SEQ ID NO. 8)

For the exchange of amino acids 54, 57 and 58 LIMA15 and LIMA16 were used:

5'-GTGTAGCAGC ACTGGCAGCT GAAATTTTA-3 (LIMA15)(SEQ ID NO. 9)

5'-AAAATTTCAG CTGCCAGTGC TGCTACACCT TCAAC-3' (LIMA16)(SEQ ID NO. 10)

For construction of the plasmid pTrpABDmut22-25-26, the double stranded PCR product was cleaved by restriction endonucleases EcoRI and PstI and for the other two mutants XbaI and PstI was used. The different fragments were ligated into pTrpABDT1T2 restricted with the same enzymes, resulting in a replacement of the wild type ABD domain gene for the three different ABDmut triple mutants. The respective mixtures were used to transform *E. coli*, strain RRIΔM15. After confirming the size of the inserts the sequences were controlled by cycle DNA sequencing (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting plasmids were denoted pTrpABDmut22-25-26, pTrpABDmut29-30-33, and pTrpABDmut54-57-58, respectively.

The DNA sequences of the three triple mutants are as follows:

ABDmut22-25-26 (SEQ ID NO. 13)

ATGAAAG CAATTTTCGT ACTGAATGCG CAACACGATG AAGCCGTAGA CGCGAATTCA TTAGCTGCTG
CTAAAGCAGC TGCTCTGAGA GAGCTCGACA AATATGGAGT AAGTGACTAT TACAAGGATC TAATCGATAA AGCCAAAACT GTTGAAGGTG TAAAAGCACT GATAGATGAA ATTTTAGCTG CATTACCTTA A

ABDmut29-30-33 (SEQ ID NO. 14)

ATGAAAG CAATTTTCGT ACTGAATGCG CAACACGATG AAGCCGTAGA CGCGAATTCA TTAGCTGAAG
CTAAAGTCTT AGCTCTGGCA GCGCTCGACG CATATGGAGT AAGTGACTAT TACAAGGATC TAATCGATAA AGCCAAAACT GTTGAAGGTG TAAAAGCACT GATAGATGAA ATTTTAGCTG CATTACCTTA A

ABDmut54-57-58 (SEQ ID NO. 15)

ATGAAAG CAATTTTCGT ACTGAATGCG CAACACGATG AAGCCGTAGA CGCGAATTCA TTAGCTGAAG
CTAAAGTCTT AGCTCTGAGA GAGCTCGACA AATATGGAGT AAGTGACTAT TACAAGGATC TAATCGATAA AGCCAAAACT GTTGAAGGTG TAGCAGCACT GGCAGCTGAA ATTTTAGCTG CATTACCTTA A

Example 10 - HSA-binding analyses of ABDmut22-25-26, ABDmut29-30-33, and ABDmut54-57-58 proteins

ABDmut22-25-26, ABDmut29-30-33, and ABDmut54-57-58 proteins were produced and purified as described in Example 2. In order to analyse the binding behaviour of the different triple-mutants a BI core 2000 instrument was used. HSA was immobilised on a sensor chip surface of a CM5 sensor chip by amine coupling to the carboxylated dextran layer. As control, a sensor chip surface containing IgG was used. This coupling was done according to the manufacturers recommendation (Biacore AB). The samples of the three proteins were run through a 0.45 μm filter and injected over the surface in a random order at a flow rate of 20 μl/min. The

concentration used was 200 nM and all samples were analysed three times. The resulting sensorgrams show that all three triple-mutants retain their ability to bind HSA (Figure 7). These results indicate that the nine positions (22, 25, 26, 29, 30, 33, 54, 57, 58) investigated in this study should be possible to subject, either simultaneously or in different combinations, to either directed or random mutagenesis in order to identify novel HSA binding ABDwt or ABDmut domains with a second activity, such as binding, catalysis or serving as substrate. In addition, residue 62 could also be used for substitutions.

Claims

1. A method of affinity separation wherein the affinity ligand is an immobilised proteinaceous ligand wherein one or more of its asparagine (Asn) residues has been modified.

2. A method of stabilising an affinity ligand by modifying one or more of its Asn residues.

3. A method of preparing a combinatorial library of protein molecules wherein the protein has been rendered less sensitive to alkaline pH by modification of one or more of its Asn residues before it is randomised.

4. A method of phage display wherein a protein expressed on the phage surface has had one or more of its Asn residues modified in a step separate to any modifications introduced in order to modify binding characteristics of the protein.

5. A method of making a stabilised combinatorial protein comprising the steps of:

a) modification of Asn residues within a protein molecule to increase stability of the protein in alkaline conditions; and

b) randomisation of the protein molecule to modify its binding characteristics.

6. A combinatorial protein wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its Asn residues.

7. A fusion protein comprising a first part wherein one or more naturally occurring Asn residues have been modified and a second part being a randomised protein

molecule selected for its specific binding properties.

8. Use of a protein molecule stabilised by modification of one or more of its Asn residues in surface display or in affinity chromatography.

9. A method or a protein or a use as claimed in any one of claims 1 to 8, wherein one or more Asn residues in said ligand or said protein are replaced with a less alkaline-sensitive amino acid.

10. A method or a protein or a use as claimed in any one of claims 1 to 9, wherein two or more Asn residues are modified.

11. A method or a protein or a use as claimed in any one of claims 1 to 10, wherein all the Asn residues are modified.

12. A method or a protein or a use as claimed in any one of claims 1 to 11, wherein Asn residues on the surface of the three-dimensional structure of the ligand or protein are modified.

13. A method or a protein or a use as claimed in any one of claims 1 to 12, wherein said Asn residues are replaced with an amino acid selected from lysine, aspartic acid and leucine.

14. A method as claimed in any one of claims 1, 2 or 9 to 13, wherein said affinity ligand is a combinatorial protein.

15. A method as claimed in claim 14, wherein said affinity ligand is a randomised protein selected by expression in a surface display library.

16. A method or protein as claimed in any one of claims 6, 7, 14 or 15, wherein said combinatorial protein is derived from an immunoglobulin molecule or a fragment or derivative thereof, staphylococcal protein A (SPA) or a fragment, domain or derivative thereof, or a DNA binding protein, or fragment or domain thereof.

17. A method or protein as claimed in claim 16, wherein said combinatorial protein is domain Z, or a derivative thereof.

18. A method or protein or use as claimed in any one of claims 1 to 17, wherein said affinity ligand or protein is Albumin-Binding Protein (ABD) or a fragment or derivative thereof.

19. Albumin Binding Protein (ABD) or fragments or derivatives thereof wherein one or more native Asn residue have been replaced by a less alkaline sensitive amino acid.

20. A nucleic acid molecule encoding a protein as defined in any one of claims 6, 7, 9 to 13 or 16 to 19.

21. A host cell expressing a protein as defined in any one of claims 6, 7, 9 to 13 or 16 to 19.

22. A fusion protein as claimed in claim 7 wherein the first part is ABD and the second part is domain Z or a derivative thereof.

template: ABD
from pTRPABDT1T2

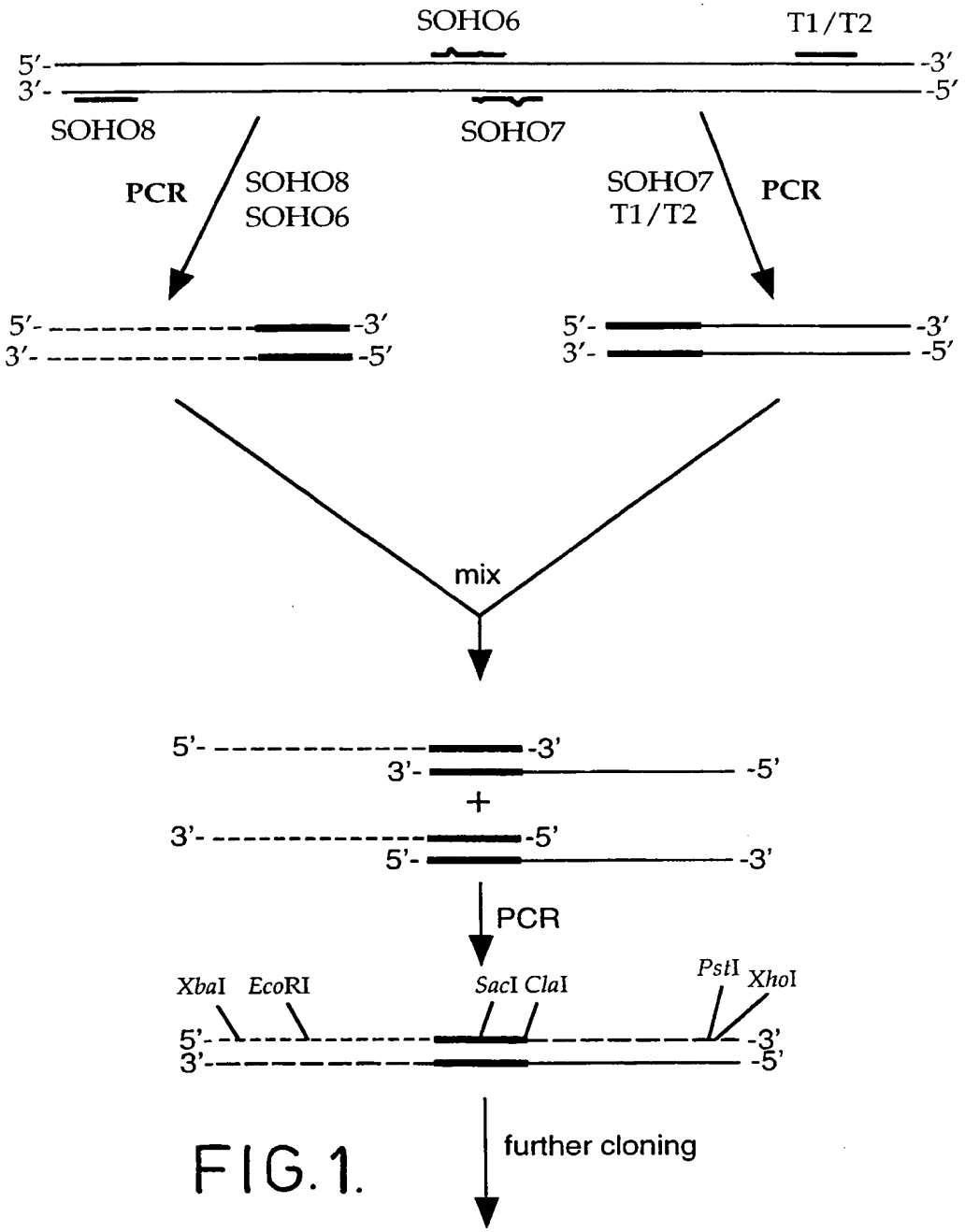


FIG.1.

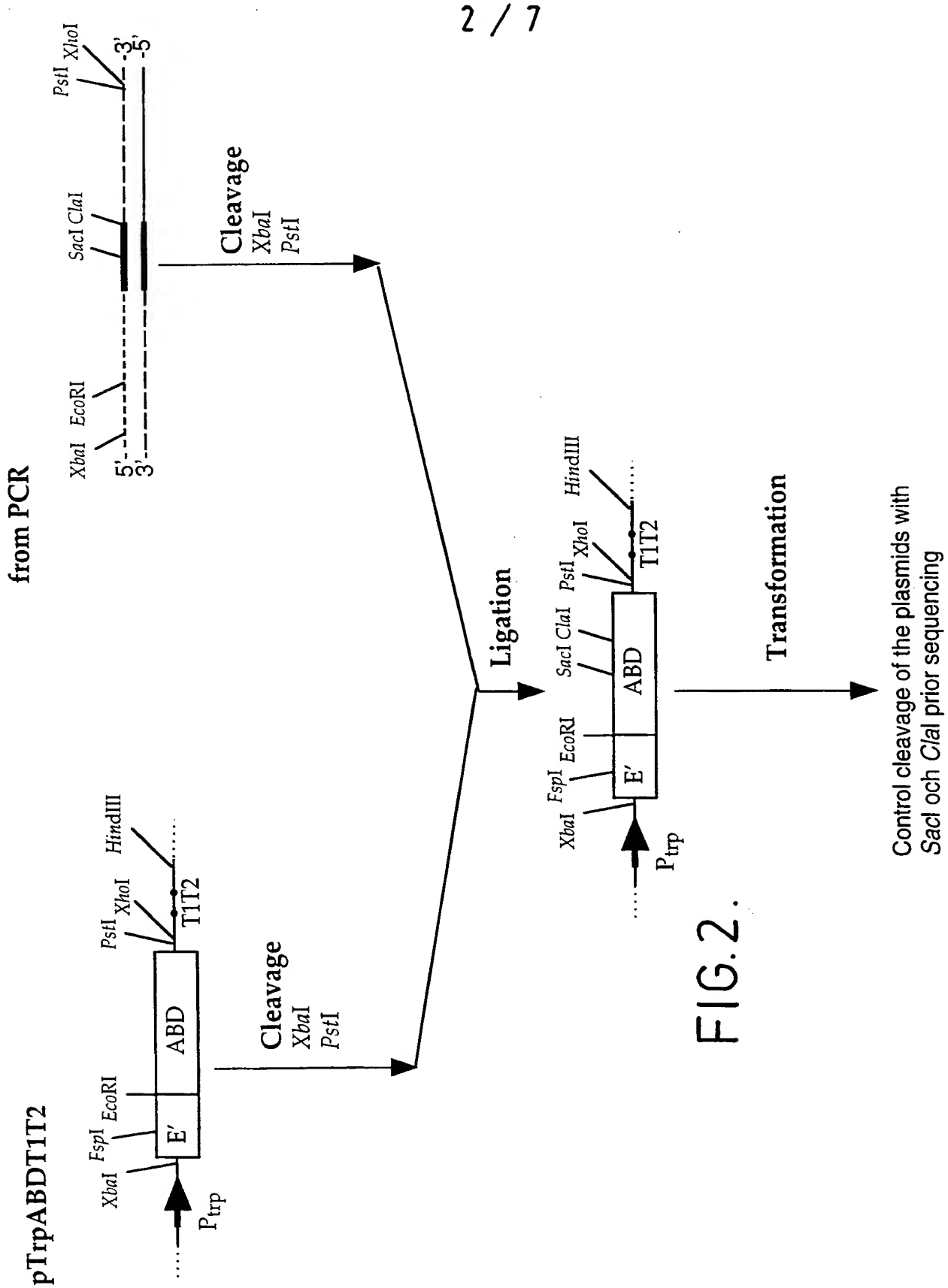
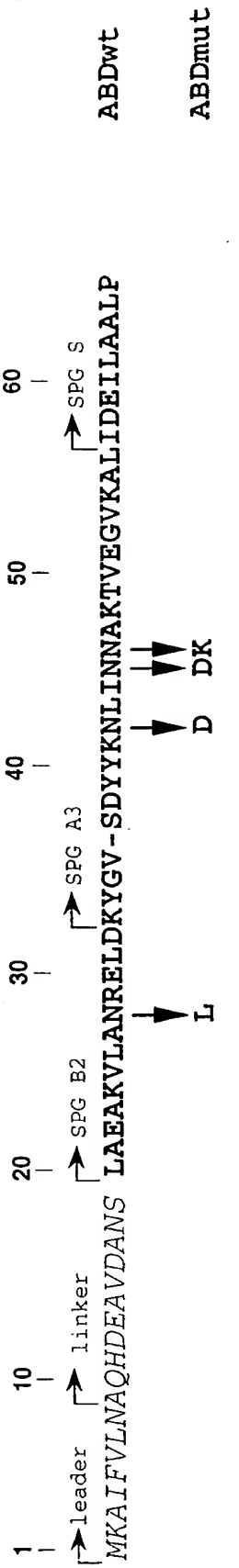

pTrpABDT1T2
XbaI
FspI
EcoRI
E'
ABD
PstI
XhoI
HindIII
T1T2
Ptrp
Cleavage
XbaI
PstI
from PCR
XbaI
EcoRI
SacI ClaI
PstI
XhoI
5'
3'
3'
5'
Ligation
Transformation
Control cleavage of the plasmids with SacI och ClaI prior sequencing

FIG. 2.

FIG. 3.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ABDmut
1
10
20
30
40
50
60
leader
linker
SPG B2
SPG A3
SPG S
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L
D
DK

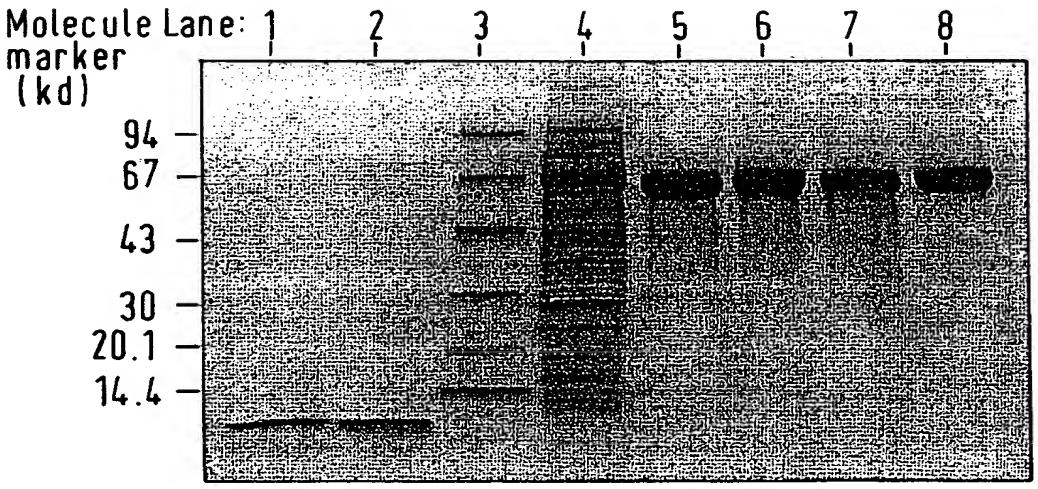
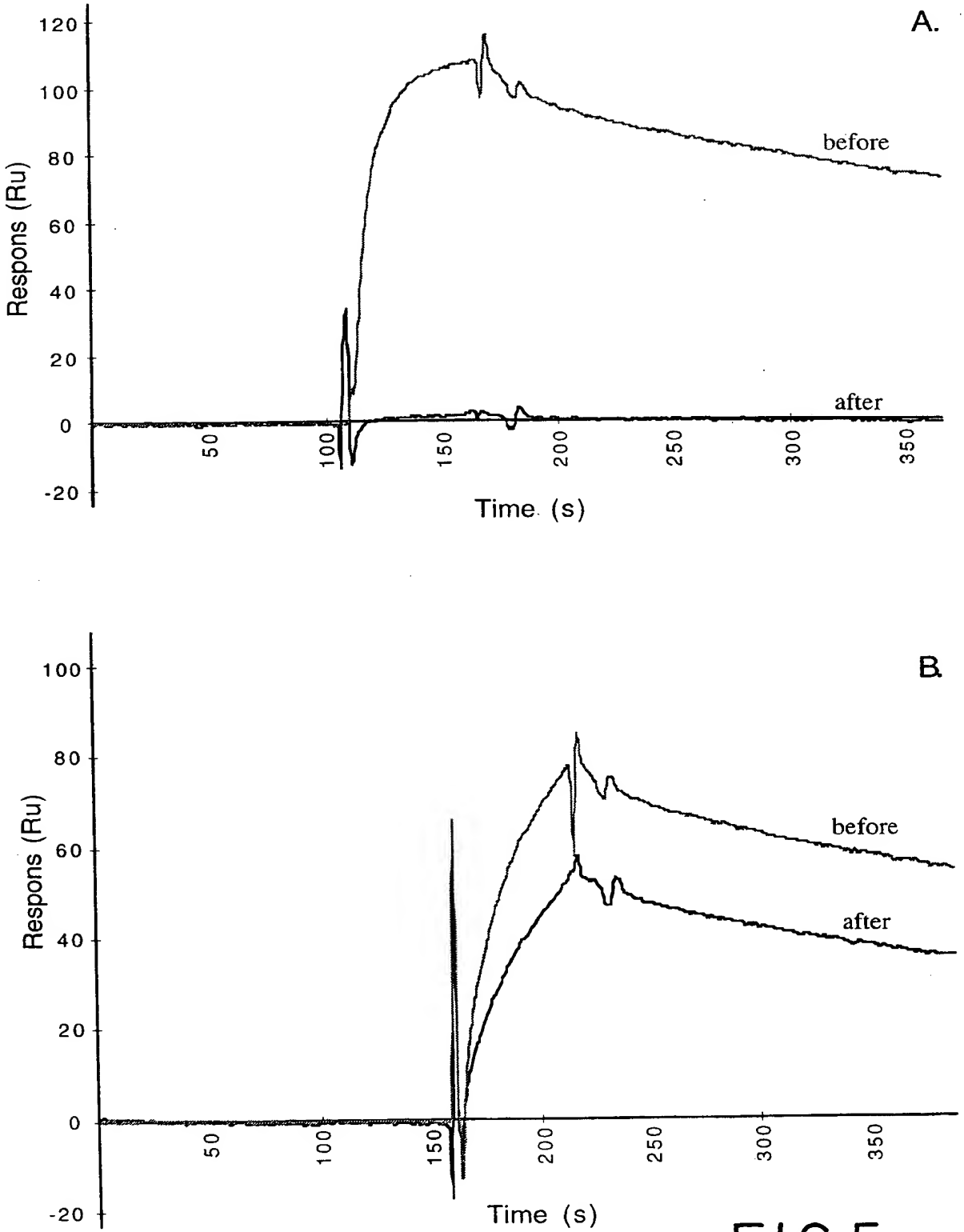


FIG. 4.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Respons (Ru)
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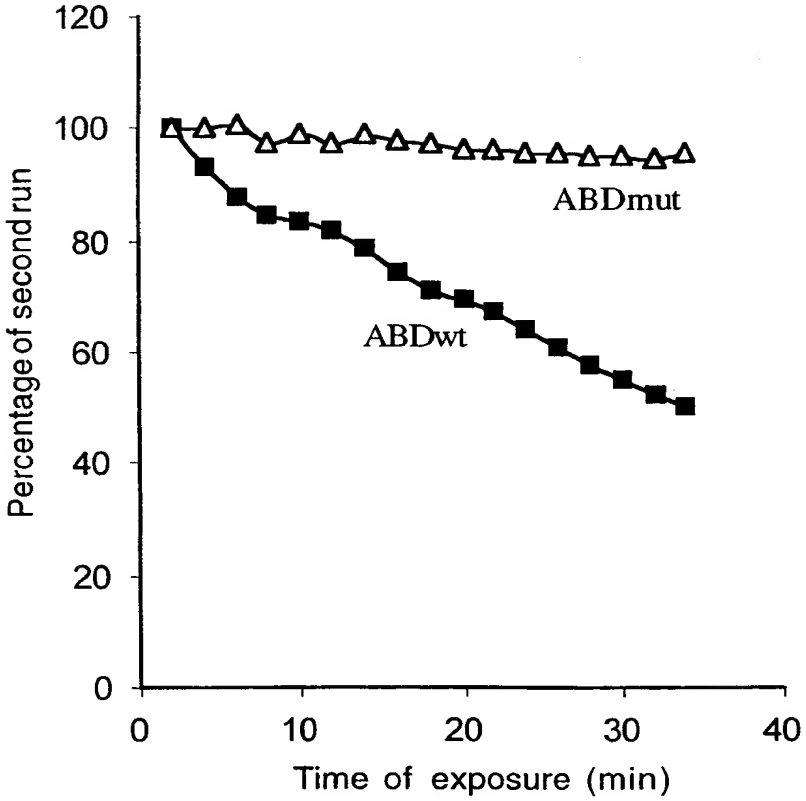
100
80
60
40
20
0
-20
50
100
150
200
250
300
350
Time (s)
before
after
B.
Respons (Ru)
100
80
60
40
20
0
-20
50
100
150
200
250
300
350
Time (s)
before
after

FIG.5.


120
100
80
60
40
20
0
Percentage of second run
ABDmut
ABDwt
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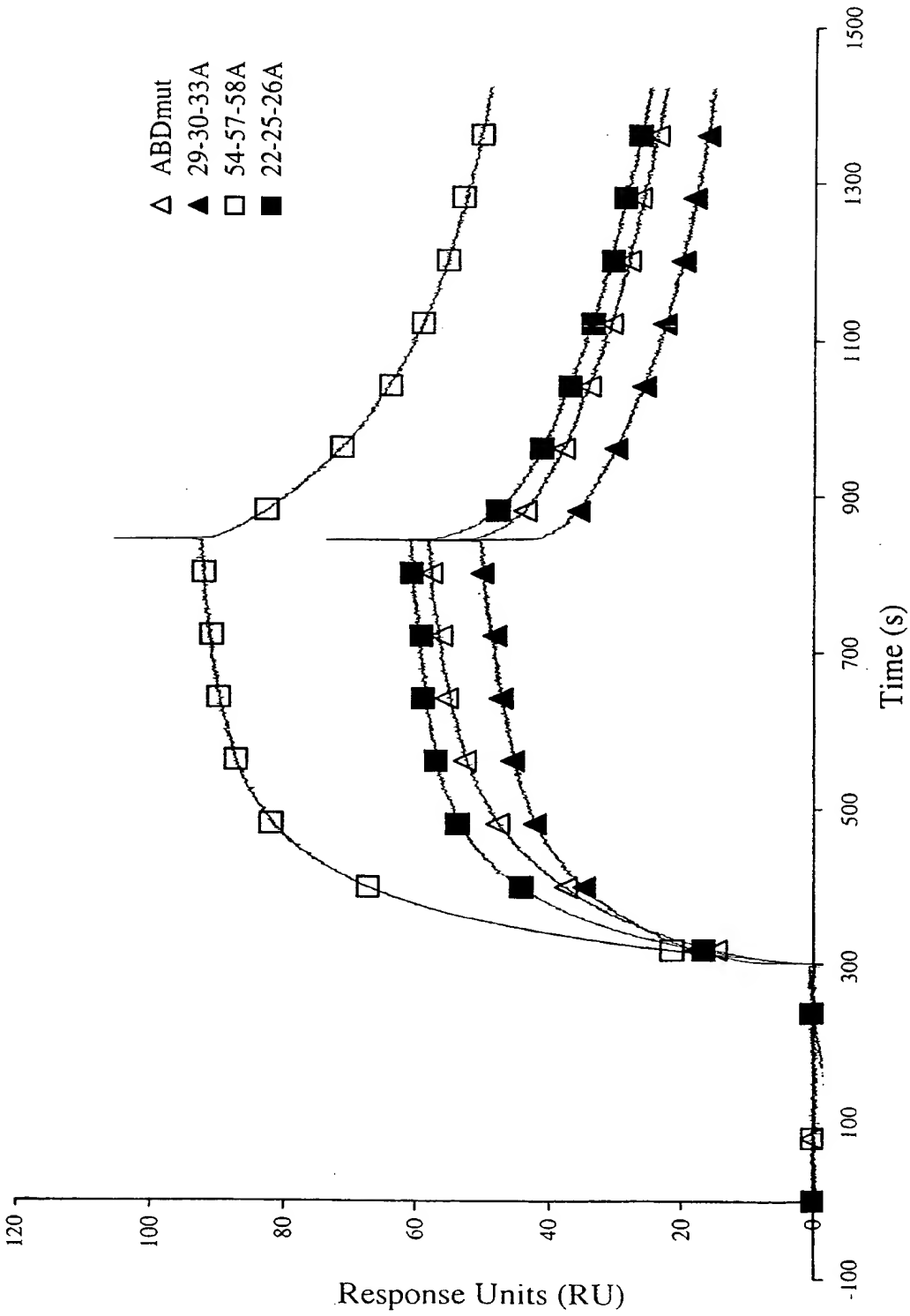
10
20
30
40
Time of exposure (min)

FIG.6.

FIG. 7.

Biosensor analysis of HSA-binding properties of different ABD protein variants



SEQUENCE LISTING

<110> Affibody Technology Sweden AB
Hober, Sophia
Uhlen, Mathias
Gardner, Rebecca

<120> A method of affinity separation and ligands for use therein

<130> 27.59.68443/001.hd

<140>
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<170> PatentIn Ver. 2.1

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Ala	Asn	Ser	Leu	Ala	Glu	Ala	Lys	Val	Leu	Ala	Leu	Arg	Glu	Leu	Asp
			20					25				30			
Lys	Tyr	Gly	Val	Ser	Asp	Tyr	Tyr	Lys	Asp	Leu	Ile	Asp	Lys	Ala	Lys
		35					40					45			
Thr	Val	Glu	Gly	Val	Lys	Ala	Leu	Ile	Asp	Glu	Ile	Leu	Ala	Ala	Leu
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Pro															
65															

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cgaaacgcct tcatccaaag tttaaaagat gacccaagcc aaagcgctaa ccttttagca 180
gaagctaaaa agctaaatga tgctcaggcg ccgaaagtag acgcgaattc attagctgaa 240
gctaaagtct tagctctgag agagctcgac aaatatggag taagtgacta ttacaaggat 300
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ttagctgcat taccttaa 198

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of ABD

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aaggatctaa tcgataaagc caaaactgtt gaaggtgtag cagcactggc agctgaaatt 180
ttagctgcat taccttaa 198

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03484

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C07K1/22 C07K14/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B NILSSON ET AL.: "A synthetic IgG-binding domain based on staphylococcal protein A" PROTEIN ENGINEERING., vol. 1, no. 2, 1987, pages 107-113, XP002133892 OXFORD UNIVERSITY PRESS, SURREY., GB ISSN: 0269-2139 the whole document --- -/--	1-22

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	07/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
A	B GUTTE: "Synthetic 63-residue RNase A analogs" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 253, no. 11, 10 June 1978 (1978-06-10), pages 3837-3842, XP002133893 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 the whole document	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/03484

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-14, 20-21
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 99 /03484

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14, 20-21

Present claims 1-15 relate to an extremely large number of possible products and vaguely defined methods. In fact, the claims contain so many variables that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear namely the method based on the products indicated in claim 16-18 and the protein indicated in claims 19 and 21. Moreover the attention of the applicant is drawn to the fact that a library as indicated in claim 5 and a protein as indicated in claim 6 are not distinguishable from any protein obtained by combinatorial chemistry, making a search for a generically defined product devoid of sense.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
27.9.68443/001		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/03484	21/10/1999	21/10/1998
Applicant		
AFFIBODY TECHNOLOGY SWEDEN AB et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 6

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/ 03484

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-14, 20-21
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14, 20-21

Present claims 1-15 relate to an extremely large number of possible products and vaguely defined methods. In fact, the claims contain so many variables that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear namely the method based on the products indicated in claim 16-18 and the protein indicated in claims 19 and 21. Moreover the attention of the applicant is drawn to the fact that a library as indicated in claim 5 and a protein as indicated in claim 6 are not distinguishable from any protein obtained by combinatorial chemistry, making a search for a generically defined product devoid of sense.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
GB 99/03484

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C07K1/22 C07K14/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B NILSSON ET AL.: "A synthetic IgG-binding domain based on staphylococcal protein A" PROTEIN ENGINEERING., vol. 1, no. 2, 1987, pages 107-113, XP002133892 OXFORD UNIVERSITY PRESS, SURREY., GB ISSN: 0269-2139 the whole document -/--	1-22

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2000	07/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Masturzo, P

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	B GUTTE: "Synthetic 63-residue RNase A analogs" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 253, no. 11, 10 June 1978 (1978-06-10), pages 3837-3842, XP002133893 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 the whole document	1-22